Tryptic Cleavage of Antibody Binding Sites from Hepatitis B Surface Antigen Particles

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SUMMARY

The sedimentation of radiolabelled 22 nm hepatitis B surface antigen particles was unaffected by treatment with either trypsin or SDS alone, but combined treatment disrupted the particulate nature of the radiolabelled material. Considerable antibody binding activity by the group-specific determinant (a) was preserved after combined SDS and trypsin treatment but was released from the bulk of the radiolabelled protein; gel filtration indicated an approximate mol. wt. of 5000 to 15000 for the released antibody binding material. This material was precipitated by concanavalin A, suggesting the presence of carbohydrate. Its serological activity was remarkably resistant to boiling and to proteolytic digestion, but was partially sensitive to treatment with 0.01 M-periodate or with mixed carbohydrases and neuraminidase, and was greatly reduced by treatment with reducing agent. These data suggest that the stability of the a determinant is due to the structure of the antibody binding site itself, rather than to involvement in the quaternary structure of the particle, and that intact disulphide bonds and carbohydrate, closely related to the antibody binding site, are necessary for the full expression of serological activity.

INTRODUCTION

There is now abundant epidemiological evidence that circulating hepatitis B surface antigen (HBsAg) in man is uniquely associated with hepatitis B virus (HBV) infection. This antigenic complex consists of a major group-specific determinant a, and at least two sets of mutually exclusive subtype determinants, d/y and w/r (Le Bouvier, 1971; Bancroft, Mundon & Russell, 1972), allowing for four possible phenotypic expressions adw, adr, ayw and ayr. Epidemiological data indicate that these phenotypes are likely to represent the expression of distinct genotypes of HBV, and thus that the antibody binding sites involved are likely to represent, or be closely related to, virus gene products (Le Bouvier, 1972). HBsAg occurs on the surface of three virus-like particle types circulating in the plasma; heterogeneous approximately spherical 22 nm forms which usually predominate, filamentous forms of similar diameter and varying lengths, and a third more complex 42 nm particle composed of an inner core and an outer lipoprotein envelope. There is evidence that passively administered antibody to HBsAg (anti-HBs), and active immunization with heat-inactivated HBsAg-positive material may confer a measure of immunity to subsequent challenge with HBV (Krugman & Giles, 1973).

Attempts to define the biochemical nature of the HBsAg antigenic sites were based
initially on the effect of various chemical and enzymatic treatments on the serological activity of 22 nm particles. Kim & Bissell (1971) reported that such activity was unaffected by treatment with 8 M-urea, acid or alkali, sodium dodecyl sulphate (SDS) up to 0.5%, most proteolytic enzymes, and heating for 6 h at 56 °C. On the other hand, activity was markedly decreased after treatment with 2-mercaptoethanol or dithiothreitol (Sukeno et al. 1972; Vyas, Rao & Ibrahim, 1972; Dreesman et al. 1973; Imai et al. 1974), although considerable activity was regained upon reoxidation unless the reduced sulphydryl groups were alkylated with iodoacetamide. Vyas et al. (1972) proposed that the conformation of the antigenic site was dependent upon disulphide bonds of the protein moiety. A progressive loss in serological activity was seen after oxidation with 0.01 M-periodate, suggesting that the integrity of the carbohydrate moiety was necessary for antibody binding (Burrell et al. 1973). More recently, Rao & Vyas (1974a) reported a loss in serological activity after combined treatment with 8 M-urea and 5 M-guanidine, and after succinylation of the ε-NH₂ group of lysine. These workers suggested that the conformational disturbance induced by disruption of covalent (disulphide) or non-covalent bonds might lead to masking of antigenic sites in intact particles without necessarily reacting with the sites themselves.

Several reports have described the release of serologically active material from 22 nm particles. After ultrasonication in the presence of 8 M-urea and 2-mercaptoethanol, protein subunits of mol. wt. 80000, 120000 and 60000 were isolated, which retained serological activity by passive haemagglutination inhibition (Rao & Vyas, 1973). In further similar studies, a peptide of mol. wt. 60000 was isolated which retained serological activity in a ‘sandwich’-type solid phase radioimmunoassay (Austria); amino acid analysis revealed a high content of tryptophan, glycine and serine, while cysteine/cystine and methionine were not detected (Rao & Vyas, 1974b). However, no data were presented on the affinity of antibody binding by this material, nor whether it contained only group-specific, subtype-specific or multiple determinants. Dreesman et al. (1973) reported the release of a serologically active fragment (mol. wt. 4000 to 12000) from HB₅Ag, after treatment with 8 M-urea and 0.1 M-dithiothreitol followed by 0.3 M-HCl; further characterization of this material has not been reported.

An alternative approach to define serologically active components of HB₅Ag has been the isolation of constituent polypeptides and glycoproteins by SDS-polyacrylamide gel electrophoresis and their use as immunogens in experimental animals. At least seven polypeptides have been described, ranging in size from 16000 to 120000 (Dreesman et al. 1975; Shih & Gerin, 1975; I. Gordon, 1975, personal communication); some, but not all, contained carbohydrate detectable by PAS staining of acrylamide gels. Dreesman et al. (1975) reported that guinea pigs immunized with many of these isolated polypeptides or glycoproteins produced specific antibody that reacted with native HB₅Ag in radioimmunoprecipitation assays; their results suggested the production of both group-specific and subtype-specific antibody. In similar work, Shih & Gerin (1975) found that all the components examined produced a humoral response to native HB₅Ag. On the other hand, I. Gordon (1975, personal communication) reported that, of 7 polypeptides examined, only one of 22000 mol. wt. produced specific anti-HB₅ in guinea pigs, whereas all polypeptides elicited a delayed hypersensitivity response when the animals were subsequently challenged with purified HB₅Ag. This work suggests that protein or glycoprotein moieties contain antigenic sites that can function as HB₅Ag-specific immunogens; however, evidence that HB₅Ag polypeptides can undergo incomplete dissociation and re-aggregation when treated with SDS and reducing agent (Mackay & Burrell, 1976) makes it difficult to attribute serological activity to determinants residing on material of clearly identifiable mol. wt.
Cleavage of HBsAg antibody binding sites

In the present report, evidence is presented that the major anti-a binding site of HBsAg can be cleaved from the bulk of the protein moiety; preliminary characterization of the released material indicates that it contains disulphide residues and carbohydrate, and that both appear to play a role in antibody binding activity. Gel-filtration suggested an approximate mol. wt. for the released material of 5000 to 15000. Further characterization of this material should lead to an immunochemical description of the antibody binding site involved.

METHODS

Purification of HBsAg. HBsAg was purified from separate samples of plasma from different antigen-positive blood donors as previously described (Burrell, 1975). Briefly, 15 to 30 ml samples of plasma were fractionated on Sepharose 6B, and HBsAg-positive fractions were pooled, concentrated and banded twice in discontinuous sucrose/caesium chloride equilibrium density gradients. The HBsAg-positive fractions were pooled and stored at 4 °C in phosphate buffered saline (PBS) containing 0.1 % sodium azide. Such preparations contained densely packed 20 to 25 nm HBsAg particles and occasional filamentous forms, and usually had an optical density at 280 nm of 0.7 to 2.0 units.

Radiolabelling of HBsAg. Preparations of purified HBsAg were iodinated with 125I by the chloramine T method, using a modification of the procedure previously described (Burrell et al. 1973). To 20 µl of a sample containing antigen (6 to 15 µg protein) were added, successively, 1 mCi of 125I (carrier-free, Radiochemical Centre, Amersham) and 20 µl of chloramine T (5 mg/ml). After 1.5 min at room temperature, 20 µl of sodium metabisulphite (12 mg/ml) and 0.5 ml of PBS containing 0.5 % bovine serum albumin (BSA) were added, and the 125I-labelled HBsAg was isolated by fractionation through a 1.0 x 20 cm column of Sepharose 6B followed by rate zonal sedimentation in a 5 to 20 % sucrose density gradient. The immunological integrity of such preparations was established by standard antiserum dilution radioimmunoprecipitation curves using rabbit antibody to HBsAg (anti-HBs), as previously described. A labelling efficiency of 5 to 25 % was routinely obtained, yielding preparations with a sp. act. of 10 to 50 µCi/µg.

Radioimmunoprecipitation assay. Double antibody radioimmunoprecipitation (RIP) assays were performed as previously described. Except where specified, samples to be assayed (100 µl) were incubated at 4 °C for 16 h in the presence of rabbit anti-HBs (Hoechst Pharmaceuticals, Hounslow, Middlesex) at a dilution of 1/1600 containing 1/200 carrier non-immune rabbit serum (50 µl). After the addition of 125I-labelled HBsAg (50 µl containing approx. 50 cts/s of 125I and 1 ng of HBsAg protein) and further incubation at 4 °C for 16 h, donkey anti-rabbit IgG (100 µl of a 1/27 dilution, Wellcome Reagents Ltd, Kent, England) was added, and the samples incubated again at 4 °C for 16 h. After centrifuging for 30 min at 2500 rev/min at 4 °C, the percentage of 125I-HBsAg precipitated was determined. The diluent used throughout was PBS containing 0.5 % BSA and 0.02 % sodium azide (RIP buffer). Sucrose gradient fractions containing SDS were examined for HBsAg serological activity by diluting 50 µl samples to 200 µl with RIP buffer and assaying as described above. Under these conditions the presence of sucrose and SDS did not interfere with the RIP assay.

Enzymes and reagents. The following enzymes and reagents were used; trypsin (2 x crystallized, salt-free, Koch-Light Laboratories, Colnbrook, England); pronase (B grade, Calbiochem Ltd, Hereford, England); mixed glycosidases from Turbo cornutus (Miles Laboratories, Slough, England); neuraminidase from Clostridium perfringens (Type VI, Sigma Chemical Company, Surrey, England); pepsin (BDH Chemicals Ltd, Poole, England); soybean
Fig. 1. Radioactivity profiles after centrifuging of $\text{^{125I}}$-labelled HBsAg through 5 to 20 % linear sucrose gradients in RIP buffer for 3.5 h at 35000 rev/min in a Spinco SW36 rotor. Samples of 500 µl were applied after treatment with (A) SDS and trypsin (●—●), (B) SDS alone (○—○), (C) trypsin alone (▲—▲) or (D) no treatment (△—△). Gradients (A) and (B) contained 0.001 % SDS. There was complete recovery of the radiolabel applied to each gradient.

trypsin inhibitor (2 × crystallized, Sigma); concanavalin A (2 × crystallized, Miles Laboratories). SDS (Specially pure, BDH Chemicals Ltd) was made up as a 10 % stock solution in distilled water and diluted for use.

To examine the effects of various treatments on the serological activity of native antigen and the released antibody-binding material, the following conditions were used; pepsin (0.1 % in 0.1 M-glycine, pH 2.5); pronase (0.1 % in RIP buffer); periodate (0.01 M in 0.1 M-phosphate buffer, pH 7.2 and 5.7, or 0.2 M-acetate buffer, pH 4.5); mixed glycosidases (0.1 %) and neuraminidase (0.05 %) in 0.05 M-acetate buffer, pH 5.5; 0.1 M-dithiothreitol in 0.6 M-tris, pH 8.2, for 1 h at 37 °C, followed by 0.1 M-iodoacetamide for 1 h at 4 °C in the dark and overnight dialysis in PBS.

**RESULTS**

**Solubilization of radiolabelled HBsAg**

Samples of radiolabelled HBsAg (20 µl or 50 µl) were incubated for 30 min at 37 °C with or without 1 % SDS; each sample was then diluted tenfold with RIP buffer, divided into two equal portions and 1 % trypsin was added to one of each pair to give a final concentration
Cleavage of HBsAg antibody binding sites

Table 1. Immunoprecipitation of 125I-labelled HBsAg after various treatments and isolation of radioactive peaks on sucrose gradients

<table>
<thead>
<tr>
<th>Fraction no.</th>
<th>7</th>
<th>11</th>
<th>15</th>
<th>16</th>
</tr>
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<tbody>
<tr>
<td>A</td>
<td>-</td>
<td>25.3% †</td>
<td>-</td>
<td>2.9%</td>
</tr>
<tr>
<td>B</td>
<td>36.0%</td>
<td>-</td>
<td>3.9%</td>
<td>-</td>
</tr>
<tr>
<td>C</td>
<td>61.9%</td>
<td>-</td>
<td>-</td>
<td>3.5%</td>
</tr>
<tr>
<td>D</td>
<td>71.4%</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* Only those fractions from each gradient corresponding to radioactive peaks were examined.
† Percentage of radioactivity precipitated by anti-HBs.

of 0.1%. After further incubation from 2 to 16 h at 37 °C, each sample was made up to 1.0 ml with RIP buffer containing 0.05% soybean trypsin inhibitor. The treatments were identified as follows: (A) trypsin and SDS, (B) SDS alone, (C) trypsin alone, (D) neither reagent. Each sample was then examined by rate zonal sucrose gradient sedimentation.

No alteration in sedimentation properties of radiolabelled HBsAg was seen after treatment with either trypsin or SDS alone, in comparison to untreated material; however, a small proportion of radioactivity was released from the particles with either treatment. In contrast, combined treatment with trypsin and SDS abolished the major HBsAg radiolabelled peak, resulting in the appearance of low mol. wt. material at the top of the gradient, and a small shoulder of intermediate sedimentation (Fig. 1). The gradient fractions corresponding to these peaks were then diluted to contain approx. equivalent concentrations of radioactivity. Samples of 0.5 ml were incubated with 50 μl of rabbit anti-HBs (1/100) at 4 °C overnight, and then with donkey anti-rabbit IgG as described in Methods (Table I). The major radiolabelled peaks after trypsin or SDS treatment alone retained immunoprecipitability comparable to that of untreated antigen. Released radiolabelled material at the top of the gradients was not precipitable, whereas the shoulder of intermediate sedimentation after combined trypsin and SDS treatment was partially precipitable; in other experiments using longer periods of trypsinization, this intermediate shoulder was abolished, and with some preparations a considerable reduction in immunoprecipitability of the major radiolabelled peak was seen after trypsinization alone (treatment C).

Assay of antigenic determinants

In the above type of experiment, serological activity would only have been detected if it had remained bound to radiolabelled material. Accordingly, a number of similar experiments were done using unlabelled purified HBsAg from different donors. The antibody binding capacity of each preparation after treatments A to D was assayed by a competitive double-antibody radioimmunoprecipitation assay. Relative measurement of antibody binding activity was possible by relating activity to the inhibition of precipitation of radiolabel observed, using a standard dilution curve of untreated HBsAg in similar assays; differences equivalent to greater than one serial twofold dilution were considered significant (Fig. 2). The degree of denaturation of antibody binding sites after various treatments was assessed by comparing the slope of dilution curves with that for untreated HBsAg (Fig. 2).

When identical samples of purified HBsAg from any one donor were treated with SDS and trypsin following procedures A to D above and assayed by double antibody RIP, serological activity was largely preserved. Some variability was noted between experiments with greatest losses usually occurring after combined trypsin and SDS treatment (A), but rarely
Fig. 2. RIP antigen dilution curves. Samples of purified HBsAg were treated (A) with SDS and trypsin (●—●) or (D) no treatment (▲—▲), and serial dilutions (100 μl) were assayed for anti-HBs binding activity by RIP. A standard antigen preparation (○---○), and 6 negative controls (100 μl of RIP buffer), were assayed similarly. The insert shows the mean value and 5 × standard deviation range obtained for the negative control assays.

Table 2. Antibody-binding activity of purified HBsAg remaining after various treatments, in comparison to an untreated sample assayed in parallel

<table>
<thead>
<tr>
<th>Experiment</th>
<th>A</th>
<th>B</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>75</td>
<td>75</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>50</td>
<td>75</td>
<td>100</td>
</tr>
<tr>
<td>3</td>
<td>20</td>
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<td>4</td>
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<td>100</td>
<td>100</td>
</tr>
<tr>
<td>7</td>
<td>25</td>
<td>75</td>
<td>75</td>
</tr>
</tbody>
</table>

* Samples of purified HBsAg were treated with SDS and trypsin (A), SDS alone (B), trypsin alone (C) or untreated (D) and assayed for antibody-binding activity by competitive double antibody RIP. The figures represent the percentage of activity remaining after each treatment in comparison to the untreated sample (D), by reading relative antibody-binding activities from a standard antigen dilution curve.

exceeding a fourfold loss when compared to the untreated (D) sample (Table 2). The degree of loss seen was not related to the source of the antigen under study. Since the slope of antigen dilution curves of treated HBsAg (A) was similar to that of untreated antigen (D; Fig. 2), it was inferred that partial denaturation of antibody binding sites was not a marked feature after such treatments.

We feel that serological activity detected in this work involved the group specific a determinant, since (1) similar results were obtained when the unlabelled antigen under study, and the labelled antigen in the assays, were of the same or opposite major subtypes (ad and ay), and (2) the rabbit anti-HBs used as first antibody in the RIP assays contained detectable anti-a activity only, at the dilution (1/1600) used in the assay.
Cleavage of HBsAg antibody binding sites

Fig. 3. Profiles of anti-HB, binding activity after centrifuging unlabelled purified HBsAg through 5 to 20% linear sucrose gradients in RIP buffer for 1-5 h at 42000 rev/min in a Spinco SW 50 L rotor. Samples of 100 µl were applied after treatment with (A) SDS and trypsin (●—●), (B) SDS alone (○—○) or (C) trypsin alone (▲—▲). Gradients (A) and (B) contained 0.001% SDS; the arrow marks the position of untreated antigen. Samples (50 µl) of gradient fractions were diluted in 150 µl RIP buffer and assayed by competitive RIP assay.

Release of serological activity from HBsAg particles

Unlabelled HBsAg particles were treated as described above (treatments A to D), analysed on rate-zonal sucrose gradients, and the fractions assayed for antibody binding activity by competitive double-antibody RIP. After SDS treatment alone (B), most of the antibody-binding activity sedimented in the same position as untreated antigen, while more rapidly sedimenting antigenic material was also usually seen (Fig. 3). After trypsin treatment alone (C), activity coincided either with the position of untreated antigen, or remained at the top of the gradient; since similar treatment of radiolabelled HBsAg always preserved the major particulate radioactive peak but in some cases reduced its immunoprecipitability (see above), we interpreted this result as reflecting the variable release of antibody binding sites from the surface of HBsAg particles after treatment with trypsin alone.

After trypsin and SDS treatment (A), antibody-binding activity was recovered only at the top of the gradients. Since radiolabel solubilized by such treatment was not immunoprecipitable (Table 1), this finding demonstrated the reproducible release of HBsAg antibody binding sites from the bulk of the radiolabelled moiety of the particles.

Preliminary characterization of antibody binding component

Previous work using intact HBsAg particles has shown a loss of serological activity after treatment with reducing agents (Sukeno et al. 1972; Vyas et al. 1972) and with 0.01 M-periodate (Burrell et al. 1973). However, since conformational changes affecting the whole particle may have accounted for such effects, we examined further the nature of the antibody binding component released from HBsAg particles by SDS and trypsin treatment.
Table 3. Comparison of the effects of various treatments on the antibody-binding activity of native antigen and the released material, determined by competitive RIP assay

<table>
<thead>
<tr>
<th>Treatment*</th>
<th>Percentage of serological activity remaining after treatment</th>
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<tbody>
<tr>
<td></td>
<td>Native antigen†</td>
</tr>
<tr>
<td>Pepsin, 3 h × 37 °C</td>
<td>50</td>
</tr>
<tr>
<td>Pronase, 16 h × 37 °C</td>
<td>100</td>
</tr>
<tr>
<td>Periodate, 3 h × 37 °C</td>
<td></td>
</tr>
<tr>
<td>pH 7.2</td>
<td>100</td>
</tr>
<tr>
<td>pH 5.7</td>
<td>20</td>
</tr>
<tr>
<td>Periodate, 16 h × 4 °C, pH 4.5</td>
<td>50</td>
</tr>
<tr>
<td>Reduction and alkylation</td>
<td>1</td>
</tr>
<tr>
<td>Reduction and dialysis</td>
<td>10</td>
</tr>
<tr>
<td>Glycosidases and neuraminidase, 24 h × 37 °C</td>
<td>100</td>
</tr>
<tr>
<td>Boiling</td>
<td></td>
</tr>
<tr>
<td>5 min</td>
<td>50</td>
</tr>
<tr>
<td>20 min</td>
<td>25</td>
</tr>
<tr>
<td>40 min</td>
<td>10</td>
</tr>
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</table>

* Detailed procedures are given in Methods.
† A preparation of HBsAg was divided in two, and one portion treated with SDS and trypsin to release antibody-binding sites.

Identical samples of purified HBsAg were treated at 37 °C with (A) or without (D) SDS and trypsin as described above; gradient analysis of a portion of each preparation for antibody binding activity confirmed that, with preparation A, all antibody binding sites had been released from intact HBsAg particles, as shown in Fig. 3. The effect of various treatments on antibody binding activity by each preparation was then examined by double antibody RIP; preparation (A) contained final concentrations of 0.05 % SDS, 0.05 % trypsin and 0.05 % trypsin inhibitor in RIP buffer. In Table 3 the antibody-binding activity remaining after the subsequent treatment shown has been expressed as a percentage of that of an identical untreated sample, by comparing relative activities from antigen dilution curves as described above (Fig. 2). It was apparent that antibody binding activity was remarkably stable to proteolytic enzymes and boiling, whereas mixed glycosidases or periodate treatment produced a small and variable reduction in antibody binding activity. A marked fall in activity occurred after reduction and alkylation. When the preparations were reduced with dithiothreitol and dialysed for 3 days against successive changes of PBS without alkylation, significant activity was regained by the preparation of native antigen, but not by the released component. With this exception, no reproducible differences were seen between native antigen and the released component, suggesting that involvement of antibody binding sites in quaternary particle structure was not of major importance in determining their susceptibility to such treatments.

Preparations of native antigen and the released component were diluted 1/4 in PBS containing 0.001 m-CaCl₂ and 0.001 m-MnCl₂ and concanavalin A was added to 0.1 %. After 4 h at 4 °C, the preparations were centrifuged for 30 min at 2000 rev/min and the supernatant fluids assayed by RIP. Antibody-binding activity was totally removed from each preparation by this treatment, whereas control samples of RIP buffer treated in the same way did not interfere with the RIP assay. This provided evidence that both native antigen and the released component contained terminal α-D-mannopyranosyl, α-D-glycopyranosyl or β-D-fructofuranosyl residues (Goldstein & So, 1965).
Cleavage of HB\textsubscript{Ag} antibody binding sites

An approximate estimate of the mol. wt. of the released material was obtained by gel filtration in a 1.6 x 15 cm column of Sephadex G-200 equilibrated with PBS containing 0.05 % SDS (Fish, Reynolds & Tanford, 1970). Internal marker proteins (2 mg ovalbumin, 2 mg lysozyme and 2 mg insulin in a total vol. of 300 ml) were incubated at 37 °C for 30 min in the presence of 6 mg SDS in an attempt to achieve binding of SDS comparable to that of the SDS-treated HB\textsubscript{Ag}; a 200 ml sample of the released material was then added, together with 100 ml of 0.04 % bromophenol blue. The total sample was applied to the column and eluted at a flow rate of 1 ml/h with PBS containing 0.05 % SDS; the position of the marker proteins was located by extinction at 280 nm, and 0.33 ml fractions were assayed by RIP. Antibody binding activity eluted as a single peak in the region of the lysozyme and insulin markers, suggesting a mol. wt. in the range of 5000 to 15,000.

**DISCUSSION**

It has previously been reported that the serological activity of HB\textsubscript{Ag} assayed by gel diffusion, was destroyed by treatment with 1-0 % SDS followed by 0-05 % trypsin, but was relatively resistant to either treatment alone (Kim & Bissell, 1971). Our results extend these observations, and demonstrate that combined SDS/trypsin treatment leads to breakdown of \textsuperscript{145}I-labelled HB\textsubscript{Ag} particles. After treatment with SDS and trypsin and in some experiments trypsin alone, significant binding activity for antibody to the \textit{a} determinant was preserved but was cleaved from the radiolabelled material, which is likely to be largely protein (Mackay & Burrell, 1976). It has previously been proposed that an external lipid shell may protect the protein moiety of HB\textsubscript{Ag} from proteolytic degradation (Le Bouvier & McCollum, 1970; Kim & Bissell, 1971); our findings are compatible with this interpretation, and suggest that with some preparations of HB\textsubscript{Ag} this protection may not be complete.

The released antibody binding material was precipitated by concanavalin A, suggesting the presence of carbohydrate; it is also likely to have contained amino-acid sequences since tryptic digestion was necessary for its release, and since its serological activity was susceptible to reducing agent. Serological activity was remarkably stable to boiling and to proteolytic digestion, which suggested that non-covalent protein-protein interactions were not involved in maintaining the integrity of the antibody binding site and that the residual protein moiety did not contain accessible proteolytic cleavage sites. Some loss in activity occurred after treatment with 0.01 M-periodate or with mixed glycosidases and neuraminidase, indicating a role for carbohydrate in the full expression of antibody binding activity; reduction and alkylation destroyed 99 % of the activity. Similar results with both the released material and untreated antigen suggested that these properties were due to the structure of the antibody binding site itself, rather than as a result of its involvement in the quaternary structure of the particles. In contrast, untreated antigen regained significant serological activity after reduction and dialysis to remove reducing agent, whereas the released material did not. This could occur if the mutual repulsion by the negative charge of bound SDS were preventing renaturation. Alternatively, if the released material were composed of more than one subunit joined by disulphide bonds and if antibody binding required these bonds intact, renaturation could be expected to proceed more efficiently where close apposition of these subunits had been maintained in the intact particle.

A mol. wt. estimate for the released material of 5000 to 15,000 was obtained by gel filtration in Sephadex G-200 in the presence of 0.05 % SDS, although this figure must remain an approximation due to the effects of the extent of binding of SDS, carbohydrate content, and incomplete unfolding of polypeptides containing intra-chain disulphide bonds.
Correlation of this material with portions of any of the polypeptides detected in SDS-disrupted HBsAg particles in the absence of trypsin digestion (Shih & Gerin, 1975; Dreesman et al. 1975; I. Gordon, 1975, personal communication; Mackay & Burrell, 1976) would be of interest. The above findings do not allow a description of the role of protein and carbohydrate in the binding of anti-HBs by the major antigenic determinant of HBsAg. Further studies, after purification of the released antibody-binding component described above, should lead to characterization of the chemical nature and configuration of the binding site by more conventional methods, and hence to information about the possible HBV gene product(s) involved in expression of serological activity. Finally, investigation of the immunogenicity of the released material should indicate if it has advantages over inactivated intact HBsAg particles in producing active immunity against hepatitis B.

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REFERENCES


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